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ANTIBACTERIAL AGENTS FROM HOPS

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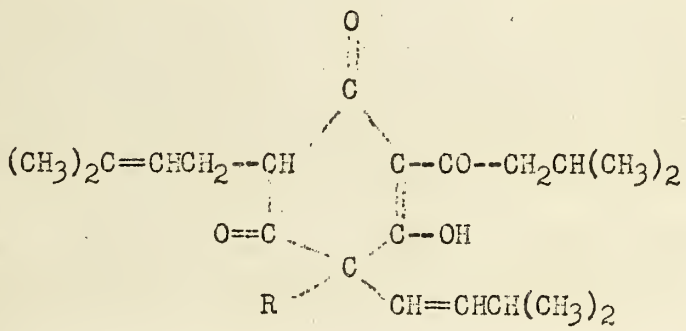
Commercial hops, the dried cones of the hop vine (Lupulus humulus), contain two antibacterial agents, lupulon and humulon. Methods of isolation of the compounds, their chemical structures, and their inhibitive action against Gram-positive bacteria have been known for many years; nevertheless, their antibiotic properties have been largely ignored in relation to animal infections. Their relatively high content in such an accessible source, together with the availability of simple methods for their isolation, prompted a survey of the antibiotic spectra of the substances. The ensuing demonstration with material supplied by this Laboratory, by Chin et al. (5)1/ at the University of California, that lupulon inhibits the growth of a virulent strain of Mycobacterium tuberculosis in vitro and exerts a pronounced effect on experimental tuberculosis infections in mice has quickened interest in microbiological, pharmacological, and therapeutic investigation of these agents.

This Laboratory is engaged in the preparation of substantial amounts of lupulon and humulon for evaluation of medical applications and commercial uses. This circular summarizes pertinent though incomplete data for research workers who may wish to cooperate in such evaluations. It will be revised periodically as additional information becomes available.

Chemical and Physical Properties

Structure: The formulas given below for lupulon and humulon had been established by 1926 (26) through the investigations of Wieland and coworkers, and Wollmer. The degradative reactions and properties of the compounds are summarized in Richter's Organic Chemistry (14). The agents have not been synthesized.



Humulon: R = -OH
$C_{21}H_{30}O_5$, MW 362.3, m.p. 55°, $[\alpha]_D$-232°, monobasic acid

Lupulon: R = -CH=CHCH$(CH_3)_2$
$C_{26}H_{38}O_4$, MW 414.3, m.p. 92°, optically inactive, monobasic acid

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1/ The numbers in parentheses refer to literature cited at the end of this circular.

Solubility and Stability: Both lupulon and humulon are fairly soluble in polar and nonpolar organic solvents such as methanol, ethanol, petroleum ether, hexane, and iso-octane. Both are only slightly soluble in neutral or acidic aqueous solution (Table 1) but are readily soluble as the sodium salts.

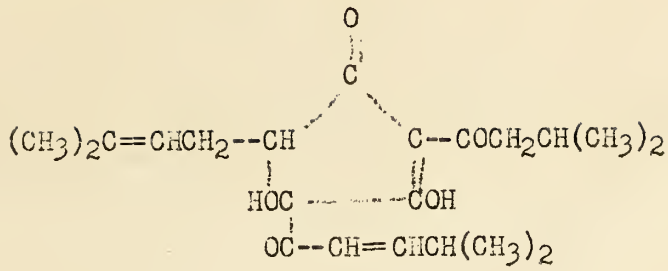
Table 1.--Solubility of lupulon and humulon in aqueous solution (adapted from a review by Walker (22)

pH	Lupulon	Humulon
	ppm.	ppm.
7.0 (phosphate buffer)	43	
6.2 " "	16	740
5.6 " "	8	244
5.2 " "		84
4.6 (acetate buffer)		20
4.0 " "		8
0.001N HCl		5
0.01N HCl		4

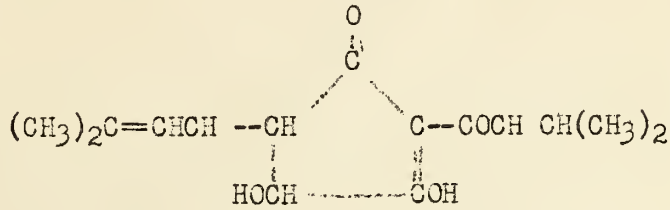
Lupulon is moderately stable to both acid and alkali. It can be partially recovered by crystallization on acidification, after it has been boiled in the presence of dilute NaOH in aqueous alcohol (21). At room temperature in the presence of air, it is very labile. In the crystalline form, it may become yellow and amorphous within a few days. This change is much slower at 5°C. than at room temperature. Storage for several months at 5°C. is accompanied by development of a characteristic odor, though color development is not marked. Lupulon crystals appear stable for months when stored in vacuo at 60°C. Recently, Lundin (11) has reported that oxidation of lupulon (and humulon) is promoted by daylight and by metal oxides, and that the oxidation is much more rapid in petroleum ether than in alcohol. We have found that the ultra-violet absorption spectra of very dilute solutions of lupulon in petroleum ether or in iso-octane change very rapidly, on standing at room temperature, but similar solutions in methanol or water have relatively stable spectra.

Michener and Andersen (12) found that the addition of 0.1 percent of ascorbic acid exerted a marked protective action on the bacteriostatic activity of lupulon steamed or autoclaved at a concentration of 4 ppm. in phosphate buffers at pH 6.5 and 8.5.

Humulon is relatively stable to acid. The lead salt is stable but the p-phenylenediamine salt slowly turns brown at room temperature in air, but not in vacuo. In aqueous solution at neutrality, or particularly in alkaline solution, humulon is transformed by boiling to an unidentified product, which is not precipitable by lead acetate but which is believed to retain some antibacterial activity (22). Quite recently, Verzele and Govaert (17) have reported the chromatographic separation of the immediate transformation product of humulon ("isohumulon"), for which they give the following structure:



They state that on boiling in methanol solution, humulon is quantitatively converted to isohumulon. On boiling with aqueous alkali, they found iso-humulon to be converted to humulinic acid, the formula of which was given as



Humulinic acid was prepared in this Laboratory by boiling humulon with 1N NaOH (25).

Michener and Andersen (12) found no loss of bacteriostatic potency against Staphylococcus aureus when 40 ppm. of humulon in phosphate buffer at pH 6.5 or 8.5 was autoclaved. However, the presence of low concentrations of ascorbic acid extended the duration of bacteriostatic action of humulon as well as of lupulon.

Assay

Determination of Lupulon and Humulon by Ultra-Violet Spectrophotometry: Although humulon and lupulon are colorless substances, both absorb radiation in the ultra-violet region of the spectrum. The differences in the ultra-violet spectra of these substances are great enough to provide a basis for spectrophotometric analysis of mixtures in solution. Figure 1 shows the absorption spectra of lupulon and humulon in methanol solution. It will be noted that both substances show major changes in spectra in going from acid to basic solution. Spectra in nonpolar solvents are rather similar to the acid forms in methanol, but the maxima are shifted to lower wave lengths and the solutions seem to be much less stable in the presence of air.

A tentative spectrophotometric method of analysis for the lupulon and humulon content of hop extracts has been developed in this Laboratory and is described below. Further work on the method of analysis and on absorption spectra of lupulon and humulon is in progress and complete details will be published soon. It has been found that a petroleum-ether extract of dried hops contains substances other than lupulon and humulon, which absorb in the spectral region of wavelengths 220 to 300 mμ and interfere with spectrophotometric methods when data from this spectral range are used. However, at longer wavelengths this interference seems to be negligible. The method described below employs optical

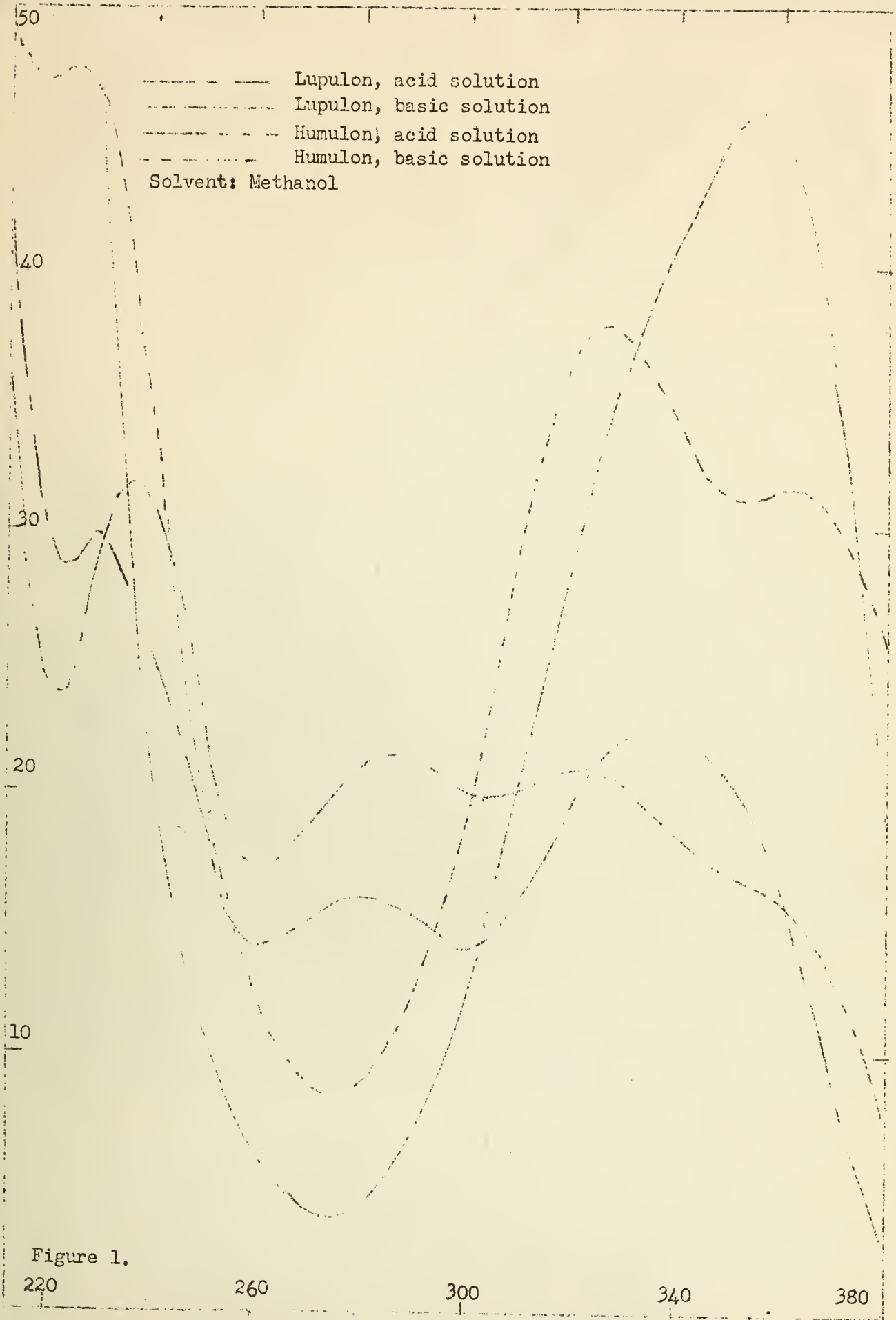

Lupulon, acid solution
Lupulon, basic solution
Humulon, acid solution
Humulon, basic solution
Solvent: Methanol
k = Specific Extinction Coefficient (l/g-cm)
50
40
30
20
10
220
260
300
340
380
Wave length in millimicrons

Figure 1.

densities at wavelengths 332 and 355 mu of an alkaline methanol solution as determined on a Beckman spectrophotometer. The procedure is as follows:

1. Dilute the petroleum-ether extract with absolute methanol so that the density falls in the range 0.5 to 1.5 at 332 mu. This density corresponds to a final concentration of the order of 30 mg. of humulon plus lupulon per liter of solution. If the original petroleum-ether solution is so dilute that less than tenfold dilution with methanol is required, an aliquot of the original solution may be evaporated in vacuo with the aid of an aspirator and the residue dissolved in methanol.

2. Add 0.2 ml. of 6N NaOH per 100 ml. of solution and take density readings at wavelengths 332 and 355 mu on the spectrophotometer, with absolute methanol as the reference liquid.

3. Calculate the results as follows:

L, H = concentration of lupulon, humulon in mg./liter.
D', D" = densities at wavelengths 332, 355 mu, respectively.
R = D"/D'
X = L/(L+H)= lupulon content fraction.

Equations (1) $X = 2.50\ R - 2.11$
(2) $L + H = 27\ D'$
(3) $L = X\ (L + H)$

The total lupulon-plus-humulon content is found from Equation 2. After X is found from Equation 1, the lupulon content is then found from Equation 3, and the humulon content by difference. These figures must be multiplied by the dilution factor to give the concentrations of the original extracts. Further work is required to establish the probable errors of analysis by the spectrophotometric method, but they appear to be of the order of 5 percent.

This method has proved very convenient in following the isolation of lupulon and humulon. The detection and assay of lupulon in body tissues by ultra-violet absorption have not been attempted, but the instability of lupulon in very dilute solutions, the presence of other ultra-violet absorbing substances, and the need for concentration of lupulon or the use of long micro-absorption cells would make such an application difficult.

Microbiological Assays: Lupulon in simple solutions can be determined by its inhibitive action on Gram-positive bacteria in turbidistatic or cup-plate tests. An inhibition of the bacteriostatic action of lupulon by blood serum, the mechanism of which is being investigated at this Laboratory by L. E. Sacks, prevents the use of such a method for determination of lupulon in blood and other tissues.

A precise microbiological method for the determination of preservative effect of hop extracts depends on inhibition of acid production by a strain of Lactobacillus bulgaricus or L. plantarum (10, 23, 24). It is interesting as an early example of the microbiological antibiotic assays now used so extensively; it proved useful for rating hops and for studying the behavior of hop constituents during brewing operations, but it is not adapted to the individual determination of humulon and lupulon in mixtures.

Other Physical and Chemical Methods for the Determination of Humulon, Lupulon, and Other Resin Fractions: Considerable effort has been spent on the determination of various resin fractions of hops for the purpose of grading hops by chemical analyses, although no precise agreement exists as to the relative importance of the various fractions. Early methods for the chemical examination of hops made the distinction between "hard resin" (soluble in ethyl ether but insoluble in petroleum ether) and "soft resin" (soluble in both ethyl ether and petroleum ether). The former is without antibiotic activity and is usually considered to be a relatively useless constituent.

Humulon ("alpha-acid" or "alpha-resin") can be precipitated from a methyl alcohol solution by the stoichiometrically equivalent weight of lead acetate. This is the basis of the gravimetric method (1). The less-well-characterized nonprecipitated portion of the soft resin is termed the "beta-resin." It contains the lupulon and transformation products of humulon and lupulon.

The method of Guthrie and Philip (8) based on the color developed with uranium acetate in hop extracts was empirically correlated by Bullis and Alderton (3) with gravimetric determinations of humulon and "soft resin." Since lupulon gives about 25 percent of the color given by humulon (Alderton, unpublished results), the method does not provide a specific determination of humulon, lupulon, or their sum.

Humulon has been determined polarimetrically after preliminary extraction of hops with methanol and transfer of the humulon to hexane in the presence of water (6, 15). For humulon, $[\alpha]_D$ = -232°, while lupulon is optically inactive.

Govaert and Verzele (7) have adapted the chromatographic separation (2) of humulon and lupulon from the dark resinous constituents of hop extracts to the determination of these substances in hops. After passage through a silical gel column, humulon is precipitated and weighed as the lead salt, whereas lupulon is extracted from the supernatant with petroleum ether and titrated with NaOH.

Lundin (11) has investigated two new methods for the independent determination of humulon and lupulon in hop extracts. One method involves an acid-base titration in a two-phase system containing water and an immiscible solvent. In such a system humulon and lupulon give different titration curves because of their different distribution coefficients. The other method involves adsorption analysis by the method of Tiselius and Claesson.

None of these methods appears to be as simple and reliable for the simultaneous determination of humulon and lupulon in hop extracts as the ultra-violet spectrophotometric method.

Isolation of Hop Antibiotics

Lupulon: Lupulon has been isolated on a kilogram scale in this laboratory by extracting freshly ground, high-quality hops with petroleum ether, concentrating the extract *in vacuo* to a thin sirup, and allowing the lupulon to crystallize spontaneously at approximately -15°C. It is then purified by repeated recrystallizations from petroleum ether and from aqueous methanol.

High-quality hops are necessary, since lupulon is one of the least stable components of hop resin, and since quantitative yields of lupulon are not obtained by this process. For example, a sample of hops which contained 5.4 percent of lupulon and 8.0 percent of humulon (by the provisional ultra-violet absorption spectra method described above) gave 3 percent of lupulon (once recrystallized), whereas another sample of hops which contained 2.0 percent of lupulon and 5.0 percent of humulon gave only a trace of lupulon crystals. For the same reason it seems preferable to extract the hops within a few days after grinding.

Since high-quality hops are resinous, they will tend to gum up the grinding mill unless the latter is kept cold. This is done conveniently by grinding an approximately equal weight of solid carbon dioxide simultaneously with the hops. (Too great an excess of dry ice should be avoided, because ground hops, having excellent insulating properties, will remain frozen and their extraction will be unduly slow.) Both the hammer-mill and shearing-blade types of mills have been used successfully. The extraction of unground hops has not been attempted, since it is probable that penetration and passage of petroleum ether through unground hop cones would be less effective, and also since ground hops pack much better.

The ground hops are extracted with petroleum ether (30-60°C.) in a column, or in countercurrent fashion in several columns. (Stainless steel or glass vessels have been used exclusively at this stage and subsequent stages.) The first extract from the column may contain 2 percent or more of lupulon plus humulon, even though the solvent cools to 5°C. by evaporation. (Most of the lupulon-humulon mixture is readily extracted, but a fraction is dissolved slowly and may be recovered if petroleum ether remains overnight in contact with the partially extracted hops.) The solvent loss by hold-up amounts to a weight about equal to that of the hops.

The petroleum ether extract is concentrated without delay <u>in vacuo</u> to a thin sirup, which is then placed at approximately -15°C. to crystallize. A rich extract may form a single porous cake so that the mother liquor can be drained off directly, or the crude crystals are filtered on a cold Büchner funnel. The crude crystals are then dissolved in warm petroleum ether (approx. 15 gm. per liter at 40°C.) and recrystallized by chilling in a dry-ice bath. The process is repeated twice, and then the lupulon is dried and dissolved in methanol (approx. 5-10 gm. per liter at 20-25°C.). The white insoluble impurity is filtered off and the lupulon is recrystallized by the slow addition of 0.1 volume of water and overnight storage at 0°C. The recrystallization from 90 percent methanol is repeated twice.

It is probably advisable to perform the last crystallizations from methanol not only to separate methanol-insoluble impurities, but also because of the evidence that lupulon is less stable in petroleum ether than in methanol; for example, we have found that the ultra-violet absorption spectrum of dilute solutions in petroleum ether and other nonpolar solvents changes rapidly (see above under Assay). Lundin (11) found catalytic oxidation to be much more rapid in petroleum ether than in alcohol. Walker (19) noted a spontaneous and irreversible redissolving of lupulon in petroleum ether.

The product consists of fine white crystals, optically inactive, with theoretical C and H contents, and melting at 92-94°. The lupulon crystals are stored in vacuo in the cold. Samples are distributed in evacuated ampoules.

The method as it stands does not permit near-quantitative recovery of lupulon; indeed, no lupulon has been obtained from low-resin hops. The mother liquors from the original crystallization contain about one-fourth of the original lupulon content (by ultra-violet absorption analysis). The first crystals, although they contain much adhering mother liquor, should be completely dissolved in petroleum ether and reprecipitated rather than washed with petroleum ether, since the latter process gives poorer recovery. Apparently lupulon is much less soluble in the pure state than in the presence of other constituents of the hop resin. High-quality hops have yielded 3 percent of once-recrystallized and 1.5 percent of 6X recrystallized lupulon, compared to the weight of the air-dry hops.

Essentially this method was used by Bungener in 1886 (4) for isolating lupulon from lupulin (the yellow resinous powder which may be shaken out of the hop cones). Lupulon has also been prepared previously (a) by direct crystallization from a petroleum-ether extract of a methanolic hop extract (from which humulon may or need not have been precipitated as the lead salt) emulsified with dilute salt water (13, 19), and (b) by extraction of such a petroleum-ether extract with weak alkali and then with 1 percent KOH, and acidifying and extracting the latter with petroleum ether from which lupulon crystallized after concentration and cooling (20). A recent method (2) for the isolation of humulon and lupulon depends on an initial chromatographic separation of these acids from other constituents of hop extracts, which are more strongly adsorped by silica gel. After passage through the column, humulon and lupulon are separated and purified by conventional means. None of these methods appears more advantageous for preparative purposes than the simple method described first.

Humulon: The conventional method for the isolation of humulon by precipitation as the lead salt has been greatly simplified in this Laboratory by making the first precipitation with o-phenylenediamine from the crude petroleum-ether extract of hops (usually from the mother liquor remaining after the crystallization of lupulon). o-Phenylenediamine was first used in 1916 by Wollner (27) for the purification of humulon lead salt. To our knowledge it has not been used previously for the direct isolation.

750 ml. of mother liquor from lupulon crystallization containing 175 gm. of humulon and 57 gm. of lupulon (by spectrophotometric analyses) was diluted with an equal volume of low-boiling petroleum ether and heated to about 40°C. To this solution was added a solution of 58 gm. of o-phenylenediamine (approximately 10 percent excess over the calculated quantity, since 1 mole of humulon reacts with 1 mole of o-phenylenediamine) in 500 ml. of boiling benzene. The phenylenediamine humulon salt crystallized rapidly and after cooling to 5°C. in the refrigerator it was filtered under nitrogen and washed with 600 ml. of cold benzene-petroleum ether (1:2). A yield of 182 gm. of salt (80 percent of theoretical) was obtained. The product was recrystallized by dissolving in 900 ml. of hot benzene followed by the addition of 1800 ml. of low-boiling petroleum ether yielding 155 gm. of pale yellow salt or a 68 percent yield based on the original humulon content.

Humulon has also been prepared by the conventional method based on precipitation with lead in methanol solution, decomposition of the lead salt with sulfuric acid, extraction of free humulon with petroleum ether, and reprecipitation as the o-phenylenediamine salt from benzene. For example, the petroleum ether extract from 8 kg. of poor-quality hops (chaffy, low lupulin) was concentrated, cooled, and 46 gm. of once-recrystallized lupulon removed (0.6 percent yield). To the mother liquor was added 6 volumes of 1 percent lead acetate in methanol plus 6 volumes of petroleum ether. The precipitated lead salt was washed in a Waring blender with a 1:1 mixture of petroleum ether and methanol to remove colored methanol-insoluble impurity. The yield of dry salt was 169 gm., equivalent to 107 gm. of humulon or 1.3 percent of the air-dry weight of the hops.

Fifty grams of the lead salt was treated with 410 cc. of 1.8 percent sulfuric acid in methanol. The lead sulfate was centrifuged off and the filtrate diluted with an equal volume of water. The humulon was extracted from the resulting suspension with 3 portions of petroleum ether. The petroleum ether extract was washed with water to remove sulfuric acid and evaporated to dryness (wt. of oil, 30 gm., 95 percent of theoretical). The humulon oil as a 50 percent solution in benzene was then treated with an equimolecular amount of purified o-phenylenediamine. The resulting crystalline salt was recrystallized twice from benzene. Two more recrystallizations from benzene were carried out under nitrogen. The product was dried and stored under nitrogen (wt. 13.4 gm.; m.p. 114-114.5°C.).

The lead-precipitation method has several obvious disadvantages, among which are: (a) the very large quantities of methanol required, (b) failure of repeated washing of the salt to remove all of the pigments, (c) failure of reprecipitation of lead humulon to give more than slight color removal, and (d) frequent emulsification with resulting loss of material on regeneration of humulon from the lead salt. Some advantages of the lead precipitation method include: (a) the specificity of precipitation of humulon, so that quantitative separation of humulon and lupulon can be attained, and (b) the stability of the lead salt which simplifies storage problems.

Antibiotic Spectra of Humulon and Lupulon

The antibiotic spectra of humulon and lupulon given in Table 2 have been determined in this Laboratory by the quantitative agar-streak dilution method of Waksman and Reilly (18). The antibiotics were dissolved in 1 percent concentration in 95 percent ethanol, and by means of aqueous dilutions, decreasing amounts were added to a series of 10-cm. Petri dishes; that is, 1.0, 0.3, 0.16, 0.1, 0.03, etc., ml. per dish. The lupulon used was a preparation recrystallized three times. Humulon was tested in two forms, namely the o-phenylenediamine salt and free humulon prepared from the above salt two hours before the test.

The test medium for bacteria and yeasts was nutrient agar (0.5 percent Difco peptone, 0.3 percent Difco meat extract, 0.5 percent NaCl, and 1.5 percent Difco agar in tap water, pH 7.0, autoclaved at 121°C. for 20 minutes). For fungi other than yeasts, potato dextrose agar was employed. Melted and cooled (45°C.) 10-ml. portions of the medium were added to plates, which were immediately and thoroughly rocked to disperse the antibiotics. After about 30 minutes, cell

Table 2.—Antibiotic spectra of lupulon and humulon

Maximum dilution (ml. per gm. of agent) producing complete inhibition of growth in agar streak test

Test organism		Antibiotic agent		
Type	Name	Lupulon	Humulon	o-Phenylenediamine salt of humulon
Gram-negative bacteria	Vibrio comma	< 3,000	3,000	3,000
	13 other spp.[1]	< 3,000	< 3,000	< 3,000
Gram-positive bacteria	Bacillus anthracis	300,000	100,000	100,000
	Bacillus cereus	300,000	100,000	100,000
	Bacillus cereus v. mycoides	1,000,000	100,000	100,000
	Bacillus megatherium	800,000	80,000	80,000
	Bacillus mesentericus	500,000	100,000	100,000
	Bacillus subtilis	1,000,000	50,000	50,000
	Corynebacterium diphtheriae gravis	100,000	10,000	10,000
	Diplococcus pneumoniae Type I	300,000	20,000	20,000
	Micrococcus conglomeratus MY	300,000	60,000	60,000
	Micrococcus lysodeikticus	300,000	60,000	60,000
	Micrococcus pyogenes v. aureus	500,000	30,000	30,000
	Sarcina lutea	100,000	30,000	20,000
	Streptococcus faecalis	500,000	30,000	30,000
Acid-fast bacteria	Mycobacterium phlei	300,000	30,000	30,000
	Myco.tuberculosis v.hominis (607)	100,000	10,000	10,000
Actinomycetes	Streptomyces coelicolor	50,000	3,000	5,000
Yeasts	4 spp.[2]	< 3,000	< 3,000	< 3,000
Other fungi	7 spp.[3]	< 3,000	< 3,000	< 3,000

1/ Aerobacter aerogenes, Alcaligenes faecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella enteritidis, Salmonella schottmuelleri, Salmonella typhosa, Serratia marcescens, Shigella dysenteriae, Shigella paradysenteriae.

2/ Cryptococcus neoformans, Saccharomyces cerevisiae, Torulopsis dattila, Zygosaccharomyces mandschurious.

3/ Alternaria citri, Aspergillus niger, Fusarium solani f. pisi, Penicillium citrinum, Rhizoctonia solani, Rhizopus nigricans, Trichoderma koenigi.